

Immunohistochemical Profile of Calponin, 14-3-3 σ and Maspin in Salivary Gland Neoplasms

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Abstract: Salivary gland tumors are a morphologically and clinically diverse group of neoplasms which may present significant diagnostic and management challenges. This study aims to investigate the expression of calponin, 14-3-3 σ , and maspin proteins in their role as myoepithelial cell markers within malignant salivary gland neoplasms, derived from myoepithelial cells (MECs). Twenty formalin-fixed, paraffin-embedded specimens of malignant salivary gland neoplasms (11 adenoid cystic carcinoma, 4 epithelial-myoepithelial carcinoma and 5 pleomorphic adenoma), were used in this study. For immunohistochemical staining, a peroxidase-antiperoxidase technique was utilized. The area fraction of immunopositivity for four different microscopic fields was measured and then used to calculate the mean area fraction. Immunohistochemical and image analysis results of the present study revealed a higher calponin, 14-3-3 σ and maspin expression in malignant pleomorphic adenoma, with intermediate expression seen in adenoid cystic carcinoma and the least expression detected in epithelial-myoepithelial carcinoma. It may be assumed that myoepithelial cells play a role in tumor development in malignant salivary gland neoplasms, where MECs exist together with luminal cells.

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1. Introduction

Salivary gland tumors (SGTs) are a morphologically and clinically diverse group of neoplasms which may present significant diagnostic and management challenges (Speight and Barrett, 2002). Histologically, this complexity has been attributed to the myoepithelial component of these tumors (Ellis and Auclair, 1996). In view of the difficulty in identifying these myoepithelial cells (MECs) with routine hematoxylin-eosin staining and even by histochemical techniques, immunohistochemistry has emerged as a useful tool in the identification of these distinct cells, contributing to improved capabilities in the detection and diagnosis of SGTs (Araújo *et al.*, 2000). To date, a variety of immunohistochemical markers have been proposed for the identification of MECs. However, as these tumors contain myoepithelial cells in different stages of differentiation, the antibodies used exhibit varying affinity for these cells, especially in comparison to MECs in normal salivary glands (Furuse *et al.*, 2005).

Calponin is a family of actin filament-associated proteins expressed in smooth muscle and non-muscle cells (Wu and Jin, 2008). It was first identified in chicken gizzard smooth muscle by Takahashi *et al.* (1986) as a 34 kDa actin and calmodulin-binding protein and later found to inhibit contraction within those cells. Calponin antibody is a sensitive immunohistochemical marker of MECs in salivary gland tumors, where it is widely expressed (Cavalcante *et al.*, 2007).

The protein 14-3-3 σ was originally characterized as a human mammary epithelial marker (Prasad *et al.*, 1992) and later rediscovered as an important molecule for cell cycle checkpoint regulation (Hermeking *et al.*, 1997). In several glandular organs, including the mammary, sweat, salivary, prostatic and pancreatic glands, 14-3-3 σ -positive cells were observed in periductal or periglandular locations, similar to the myoepithelial cells (Hermeking *et al.*, 1997).

Maspin (mammary serine protease inhibitor) belongs to the serine protease inhibitor (serpin) family. This serpin family comprises a large protein group with diverse biological functions (Araújo *et al.*, 2000). Maspin expression has been demonstrated in multiple tissues, including breast, prostate, and lung epithelia, as well as in stromal cells of the cornea (Prasad *et al.*, 1992; Hermeking *et al.*, 1997; Nakajima *et al.*, 2008). In general, maspin is expressed in large quantities in myoepithelial cells. Thus, some authors (Reis-Filho *et al.*, 2001; Ngamkitidechakul *et al.*, 2001; Reis-Filho *et al.*, 2002; Yatabe *et al.*, 2004; Schwarz *et al.*, 2008) suggested the use of maspin as a myoepithelial cell marker.

This study was conducted to evaluate the immunohistochemical expression pattern of calponin, 14-3-3 σ and maspin as myoepithelial cell markers in malignant SGTs, in which MECs share in their histogenesis.

2. Material and Methods

Twenty formalin-fixed and paraffin-embedded specimens of malignant salivary gland tumors were collected in June 2012 from the archives of the Pathology Department, National Cancer Institute, Cairo University. Eleven tumors were diagnosed as adenocystic carcinomas (ACC), four were diagnosed as epithelial-myoeplithelial carcinomas (EMC) and five were diagnosed as malignant pleomorphic adenomas (MPA). Histopathological examination for the collected cases was additionally performed to confirm the diagnosis.

Immunohistochemical staining was carried out using the biotin-streptavidin method, utilizing antibodies against calponin, 14-3-3 σ and maspin. Immunostained sections were examined by light microscopy. Photomicrographs were captured at a magnification of 20X, using a digital video camera (Olympus, C5060, Japan) mounted on a light microscope (Olympus, BX60, Japan). Images were then transferred to the computer system for analysis. The captured microscopic fields were analyzed using standard image analysis software (Image J, 1.37v, NIH, USA). Images were automatically corrected for brightness and contrast, and then converted into 8-bit gray scale type. Phase color-coding of the desired areas was automatically performed. Threshold was adjusted to select for immunopositive cells. Data was then tabulated in Microsoft Excel sheets (Microsoft Office 2007®). The mean area fractions MAFs were calculated.

3. Results

All examined cases of adenoid cystic carcinoma showed positive calponin, 14-3-3 σ and maspin immunoreactivity, with the exception of two cases of solid variant tumor which were maspin immunonegative. Positive immunoreactivity for calponin was mainly seen in MECs along the periphery of the tubular, cribriform and solid growth patterns and in some of the malignant cells lining the pseudocystic spaces of cribriform patterns. Immunoreactivity was nuclear and cytoplasmic in localization. The basaloid cells were found to be immunonegative (Figure 1).

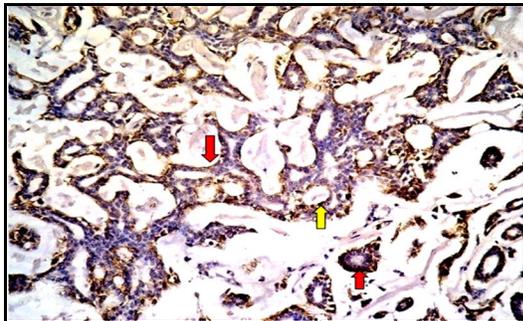


Figure 1. Adenocystic carcinomas showing calponin immunopositivity of the myoepithelial non-luminal cells in tubular and cribriform patterns (red arrows) and the myoepithelial cells lining the pseudocystic spaces of cribriform patterns (yellow arrow). Note the immunonegative basaloid cells (original magnification x 20).

For 14-3-3 σ , strong cytoplasmic immunopositivity was seen in focal areas of the lesion, while the rest of the lesion was found to be only weakly immunoreactive (Figure 2).

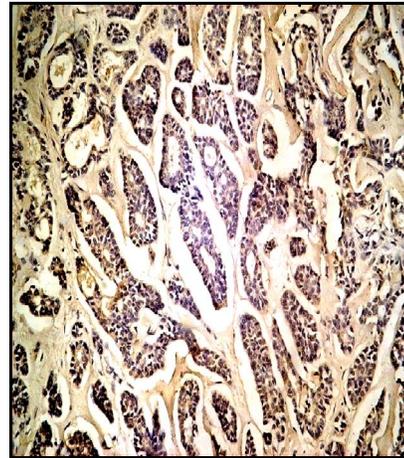


Figure 2. Adenocystic carcinomas showing tubular, cribriform and solid growth patterns with cytoplasmic 14-3-3 σ immunoreactivity (original magnification x 20).

In ACC, 10 cases of cribriform and tubular patterns demonstrated cytoplasmic maspin immunostaining (Figure 3).

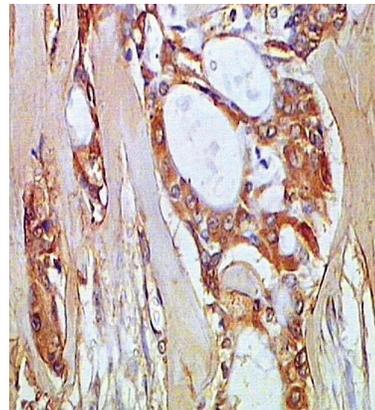


Figure 3. Adenocystic carcinomas showing cytoplasmic maspin immunoreactivity of the basaloid neoplastic cells. Note the negatively-immunostained myoepithelial cells (red arrow) (original magnification x 40).

Two cases of solid pattern were negative for maspin. Myoepithelial cells were immunonegative.

In epithelial-myoepithelial carcinoma, all the examined cases showed positive calponin, 14-3-3 σ and maspin immunoreactivity. Furthermore, positive immunoreactivity for calponin was mainly seen in the malignant MECs, while only a few malignant epithelial cells showed positive immunoreactivity. Immunopositivity was found to be either cytoplasmic or both cytoplasmic and nuclear in localization (Figure 4).

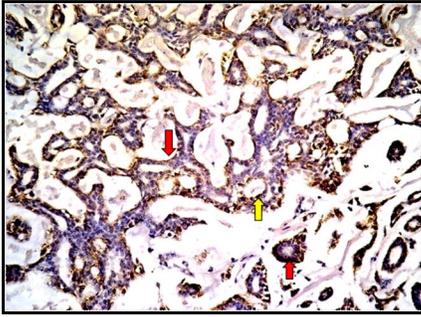


Figure 4. Epithelial-myoepithelial carcinomas showing calponin immunopositivity in malignant epithelial cells (red arrows) and calponin immunonegativity in malignant myoepithelial cells except for a few immunopositive cells (yellow arrow) (original magnification x 40).

For 14-3-3 σ , immunoreactivity was seen in the malignant myoepithelial and epithelial cells. Malignant MECs showed either cytoplasmic or both cytoplasmic and nuclear immunoreactivity while the malignant epithelial cells showed cytoplasmic immunoreactivity (Figure 5).

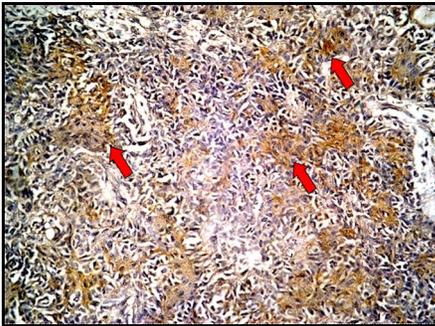


Figure 5. Epithelial-myoepithelial carcinomas showing cytoplasmic 14-3-3 σ immunoreactivity in malignant epithelial cells (red arrows) (original magnification x 40).

Regarding maspin immunoreactivity, some neoplastic cells demonstrated cytoplasmic and nuclear (either the whole nucleus or confined only to the nuclear membrane) positive immunostaining while other cells were immunonegative (Figure 6).

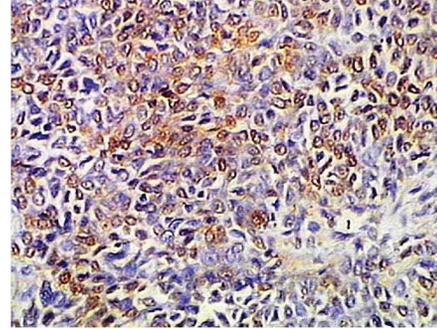


Figure 6. Epithelial-myoepithelial carcinomas showing positively and negatively immunostained myoepithelial cells (original magnification x 40).

In malignant PA, all of the examined cases showed positive calponin, 14-3-3 σ and maspin immunoreactivity. Immunopositivity was seen in epithelial cells, spindle-shaped and plasmacytoid MECs, irrespective of tumor arrangement into sheets, cords or duct-like structures. Some of the stromal cells were immunopositive. This immunopositivity was either cytoplasmic or both cytoplasmic and nuclear in localization (Figure 7-9).



Figure 7. Malignant pleomorphic adenomas showing calponin immunoreactivity in plasmacytoid myoepithelial cells (red arrow), epithelial cells (yellow arrow), and spindle-shaped myoepithelial cells (green arrow) (original magnification x 40).

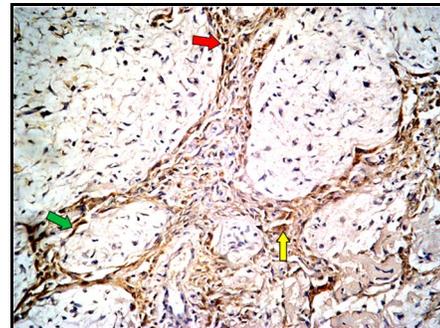


Figure 8. Malignant pleomorphic adenomas showing 14-3-3 σ immunoreactivity in plasmacytoid myoepithelial cells (red arrow), epithelial cells (yellow arrow) and spindle-shaped myoepithelial cells (green arrow) (original magnification x40).

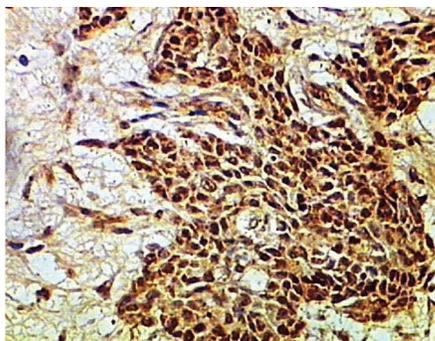


Figure 9. Malignant pleomorphic adenomas showing maspin immunostaining of the malignant epithelial cells. Note positive (red arrow) and negative (yellow arrow) myoepithelial cells (original magnification x 40).

4. Discussions

Neoplastic myoepithelium is considered to be the key cellular participant in morphogenetic processes responsible for the variable histological appearances of many salivary gland tumors. Nevertheless, there are still controversies regarding its participation in some types of salivary gland neoplasms. This controversy has been largely due to the difficulty in fully characterizing the wide spectrum of morphologic and immunophenotypic expressions of neoplastic myoepithelium when compared with the normal counterpart. In recent years however, our understanding of the phenotypic, immunophenotypic, ultrastructural and biochemical properties of myoepithelium has evolved dramatically (Adnan and Richard, 2004).

H1calponin is a basic actin-binding protein that is abundantly expressed in smooth muscle cells (SMCs) and involved in smooth muscle contraction by inhibiting actomyosin magnesium ion activated adenosinetriphosphate (Kaneko *et al.*, 2002). Calponin antibody is a sensitive immunohistochemical marker of MECs in salivary gland tumors that exhibits a primarily intense expression (Cavalcante *et al.*, 2007). It has been shown to be a highly sensitive marker of MECs in human mammary tissue and tumors (Martin de las Mulas *et al.*, 2004). Of note, h1calponin was recently reported to play an active role in tumor suppression as well (Kaneko *et al.*, 2002).

The 14-3-3 σ protein was demonstrated as a new adjunct antibody in the characterization of MECs and myoepithelial lesions and as a possible prognostic factor in breast cancer patients (Simpson *et al.*, 2004). Similar to other myoepithelial markers, 14-3-3 σ protein has not been found to be specific for MECs.

One of the first regulatory mechanisms identified for maspin involved p53 signaling. Regulation of maspin by p53 could help explain the

role of p53 in cell invasion and metastasis. Furthermore, cancer cells expressing mutant p53 were found to be more likely to metastasize, partly due to the inability to upregulate the maspin gene (Bailey *et al.*, 2006). In addition, high maspin levels were associated with an increase in apoptosis and a reduction in cell invasion. It was also suggested that maspin has an inhibitory effect on tumor induced angiogenesis (Cher *et al.*, 2003), cell motility, invasion and metastasis (Navarro *et al.*, 2004).

In ACCs, positive immunoreactivity for calponin (12.66 pixel²) was principally observed at the periphery of cribriform and tubular patterns and scattered in the solid pattern. It was also seen within some cells lining the cystic-like spaces of cribriform patterns. These findings are consistent with the distribution of MECs in ACCs and with findings described by Prasad *et al.* (1999), Ogawa *et al.* (2000), Furuse *et al.* (2005) and Cavalcante *et al.* (2007). For 14-3-3 σ , immunoreactivity (9.19 pixel²) was seen in the neoplastic myoepithelial and epithelial cells in some areas of the lesion, while the rest of the lesion demonstrated negative immunoreactivity. Uchida *et al.* (2004) observed that 14-3-3 σ was hardly detected in the neoplastic MECs and faintly expressed in the neoplastic duct cells of salivary ACC. Of note, this marker is known to be often down-regulated in ACC, and this down-regulation could play critical roles in the neoplastic development and radiosensitivity of ACC. Maspin (17.5 pixel²) was expressed only in the malignant epithelial cells of the tubular and cribriform patterns, confirming their low grade behavior. Maspin immunonegativity in the solid variant is in agreement with the findings of Navarro *et al.* (2004), partially explaining its aggressive behavior. MECs were also maspin immunonegative, which is also in accordance with Navarro *et al.* (2004). Normally maspin is expressed in high amounts in MECs, so loss of maspin in MECs of ACC might indicate the role of these cells in the malignant transformation and histogenesis of this neoplasm.

In EMC, calponin immunoreactivity (6.006 pixel²) was observed in the neoplastic MECs, and this is in agreement with Furuse *et al.* (2005) 4 and Prasad *et al.* (1999). Only a few neoplastic epithelial cells showed positive immunoreactivity in our study. These cells could be epithelioid MECs, as the MECs have the potential to undergo divergent differentiation giving rise to different morphologic cell types. On the other hand, 14-3-3 σ immunoreactivity (6.59 pixel²) was seen in the neoplastic epithelial and MECs. To our knowledge, there is no report describing the expression of 14-3-3 σ in EMC. Several studies stated that EMC should be classified as a high-grade malignancy (Guangyan *et*

al., 2003; Savera *et al.*, 2000; Ohba *et al.*, 2009). This is consistent with our results, which demonstrated very low maspin expression (6.2 pixel²).

In malignant PA, calponin immunopositivity was observed in neoplastic cells forming strands, masses and duct-like structures along with some of the stromal cells. MECs were also immunopositive. Melissa *et al.* (Melissa *et al.*, 2005) stated that high levels of calponin (16.24 pixel²) may affect cancer MECs: contrary to acting as tumor suppressors, it may cause these cells to induce growth, migration and invasion. 14-3-3 σ and maspin also showed a similar staining pattern. High levels of 14-3-3 σ expression (13.84 pixel²) might provide protection against apoptosis, as these levels can lead to sequestration of pro-apoptotic factors, such as BAD and BAX (Hermeking, 2003). Therefore, this protein may play a key role in carcinogenesis through multiple mechanisms and its role in migration, metastasis and invasion could be tissue- or cancer type-dependent (Li *et al.*, 2009). Maspin was also found to be expressed in high levels (38.5 pixel²). Once again, MECs may have lost their tumor suppressing properties as a result of genetic mutation (Umekita *et al.*, 2002).

Taken together, the results of this work demonstrate that myoepithelial cells play a major role in the development of malignant salivary gland tumors, where MECs exist together with luminal cells. Higher levels of calponin may predict a more favorable prognosis due to its tumor suppressive actions, but it remains unclear how regulation of 14-3-3 σ contributes to the development of neoplasia. Furthermore, decreased maspin expression in MECs could indicate the role of these cells in malignant transformation.

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